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GENETIC DIVERSITY AMONG *JUGLANS REGIA* AND *PRUNUS DULCIS* GENOTYPES USING RAPD AND ISSR MOLECULAR MARKERS

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Abstract

Genetic variability among one genotype of walnut (*Juglans regia* L.) and three genotypes of almond (*Prunus dulcis* L.) were studied by using molecular characterization. Two markers generated variability among studied cultivars RAPD & ISSR. RAPD marker produce a total number of bands 63 consists of 27 monomorphic and 36 polymorphic bands, generated polymorphism as 57.14%. Among the resulted polymorphic bands, a13 unique bands were observed. Whenever, ISSR marker generates 68 total bands with 17 monomorphic bands and 51 polymorphic bands, from these polymorphic there were 17 unique bands. Obviously, ISSR gave polymorphism among the examined plants with percentage of 75%. Data of molecular attributes computed and illustrated highest similarity coefficient among hard and scrub almond genotypes (sweet almond) and also was represented in one cluster separated the studied 4 genotypes into two groups; one group includes walnut genotype and other group includes; sweet almond (scrub hard) and bitter almond. ISSR marker produces similarity of 75% higher than the RAPD marker 57.14%, so, ISSR molecular markers could be the suitable method to differentiate among walnut and almond genotypes rather than RAPD markers.

Key words: Genetic polymorphism*, Juglans regia*, *Prunus dulcis*, RAPD-PCR and ISSR– PCR

Introduction

Cultivated *Juglans regia* L. is a monoecious and dichogamous species, considered as the most edible nuts and economic species in family Juglandaceae and called as *Persian walnut* (Tabasi *et al*., 2020). *Persian walnut* is wildly distributed all over the tropical and temperate regions of the world from Americans and Europe to Asia (APG IV, 2016). Its fame due to the highest nutritive value that contain and rich in proteins, minerals and fats, in addition to it considered as one of the most natural source of energy (Pereira *et al*. 2008). It has an effective effect on human health derived from its high antioxidant capacity, high concentration of fatty acids, as well as various vitamins of group B (Anderson *et al*., 2001). Oil prepared from the walnut nut is immensely beneficial for women suffering from menstrual dysfunction (Ros and Mataix, 2006).

The almond*; Prunus dulcis* L. (Batsch) belong to family Rosaceae, it is cultivated tree native to the Middle East and South Asia (Colic *et al*., 2012). It well known that, Almond distributed in subtropical Mediterranean climate therefore, the almond production is concentrated in some regions; Mediterranean and Asian countries (Simsek and Demirkiran, 2010). Moreover, Almond characterized by its resistance to salinity and drought, so farmers prefer to cultivate it in the most cultivated areas in the world (Sorkheh *et al*., 2011).

Economic importance to almond due to its highest content from oil and fatty acid. Total dry weight of almond contains a percentage of oil varied from 48% to 67%. Their composition varied significantly from genotype to another intra the same species. These oils includes both of fatty acids and linoleic acids that considered the major components in the oil, vartion from 12% to 27% was recorded with fatty acids but it was ranged from 63% to 78% oleic acids (Kodad and Socias, 2008).

Genetic variation for certain species is the key for

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knowledge of management and future using of this species and/or its germplasm in plant breeding resulting in newly cultivars (Bernard *et al*., 2018). Walnut and almond are highly different in morphological and taxonomical features. So, studying the evaluation of genetic diversity among different species based on; phenotypic and genotypic, so, molecular markers could be used to give definite information about their genetic routes of each individual in the same species (Ebrahimi *et al.,* 2011). Actually, morphological traits only not sufficient for accurate identification of specific plant species (Kumar, 1999; Mahmoodi *et al.,* 2013), morphological traits should be confirmed by molecular characterization which resulted in well classification and identification. The DNA markers are the efficient way has been suggested for the illustration of genetic variability and similarity within and among the genotypes (Kumar *et al.,* 2019). There are many molecular markers were used to differentiate among the walnut and almond genotypes such as isozymes (Viruel *et al*., 1995), RAPD (Gouta *et al*., 2008), ISSR (Martins *et al*., 2003), RFLP (Tabasi *et al*., 2020; Wang *et al*., 2017), AFLP (Martins *et al*., 2001) and SNP (Wu *et al*., 2008). The common molecular marker was used in genetic variation among walnuts was ISSR (Mahmoud and Abd El-Fatah, 2020).

Zhaobin *et al*., (2016) studied the biodiversity among four Chinese wild almonds and two cultivars of *Amygdalus communis* L. using SRAP marker. Studies of the genetic genetic polymorphism among different cultivars of walnut and almond were little. Due to the low number of the articles studied the genetic polymorphism among the walnut and almond; we aimed to study the genetic diversity among different genotypes of almond and of walnut using the molecular maekers RAPD-PCR and ISSR-PCR. This type of study will be important clues for plant species conservation and management and breeding for these important crops.

Table 1: List of RAPD and ISSR primers name, size ranges and their sequences.

Marker	Primer name	Sequence	Size Range(bp)
RAPD	OP-A2	5´GTG ATC GCA G 3‘	200-500
	OP-A07	5´GAA AGG GGT G 3‘	130-500
	OP- B7	5´GGT GAC GCA G 3‘	270-1300
	OP-B11	5‘ GTA GAC CCG T 3‘	250-1300
	OP-C04	5- -CCG CAT CTA C- 3-	280-900
	OP-C9	5´CTC ACC GTC C 3‘	250-1300
ISSR	14A	5 CTC TCT CTC TCT CTC TG 3‘	100-800
	49A	5‘CAC ACA CAC ACA CAC AG 3‘	400-1100
	HB-9	5‘ CAC ACA CAC ACA CAC A (AG) T 3‘	150-1300
	HB-10	5‘ $(GAG)_2(AGA)_2$TGC CC3‘	150-1700
	HB-12	5‘ TCT CTC TCT CTC TCT CA 3‘	100-1000
	HB-15	5´GTG GTG GTG GC 3‘	270-900

Table 2: Number of total bands, polymorphic bands and percentage of polymorphism of each primer generated.

Parameter	RAPD	ISSR
Studied taxa	4	4
No. of primers	6	6
Marker range (bp)	130-1300	100-1300
Total bands	63	68
Monomorphic bands	27	17
Polymorphic bands	36	51
No. of unique bands	13	17
% Polymorphism	57.14%	75%

Materials and methods

Extraction of DNA

Young leaves of one genotype of walnut (*Juglans regia* L.) and three genotypes of almond *Prunus dulcis* L. (Batsch); one genotype of bitter almond and two types of sweet almonds; scrub Almond and hard almond were extracted using CTAB buffer (Kriz¡man *et al.,* 2006) and the resulted DNA was purified and both of purity and concentration of the extracted DNA were determined using Nano drop.

PCR Reaction

Six primers of RAPD and six primers of ISSR were used to study the genetic diversity; their name, bands size and sequences were shown in table 1. In the PCR, reaction mixture was 20µl containing 10µl of 2x TOP simple™ DyeMIX-HOT, 5µl of each Primer, 1µl of genomic DNA and volume was completed into 20 µl with sterile water. The PCR schedule as; one cycle at 95ºC for 10min followed by 40 cycles of; 94ºC for 30 sec, 30ºC for 1min for each primer, 72ºC for 2min and a final incubation at 72ºC for 5min. The PCR products were separated on a 1.5% agarose gel and the gel was visualized using DNA gel documentation according to (Mandal *et al*., 2014).

Data analysis

All gels were photographed and analyzed using Bio-Rad video documentation system, Model Gel Doc 2000. Only distinct, reproducible, well-resolved fragments were scored as present (1) or absent (0) for each of the RAPD and ISSR markers. Dendrogram of cluster analysis and genetic similarity were used to illustrate and estimate the genetic distances and relationships among studied genotypes using the SYSTAT version

7.0 software (Wilkinso, 1997).

Results

For RAPD analysis

Six primers were used with molecular size ranged from 130-1300bp, about 63 total bands were generated with 27 monomorphic bands and 36 polymorphic bands as shown in table 3. Primer OPC9 produced highest number of total bands as 18 with 11 monomorphic bands and seven polymorphic bands. Primer OPA2 showed lowest number of total bands as six bands with three monomorphic bands and three polymorphic bands as illustrated in table 2. Primers OPB7 and OPC4 gave highest number of unique bands as four bands. OPB7 produced three unique bands for bitter almond at size 600, 1000 & 1300 bp. and one band unique characteristic for sweet almond (hard almond) at size 350 bp. Primer OPC4 generated three unique bands characteristic for walnut at sizes 280, 300 & 400 bp and one unique band for sweet almond genotype (scrub almond). Regarding,

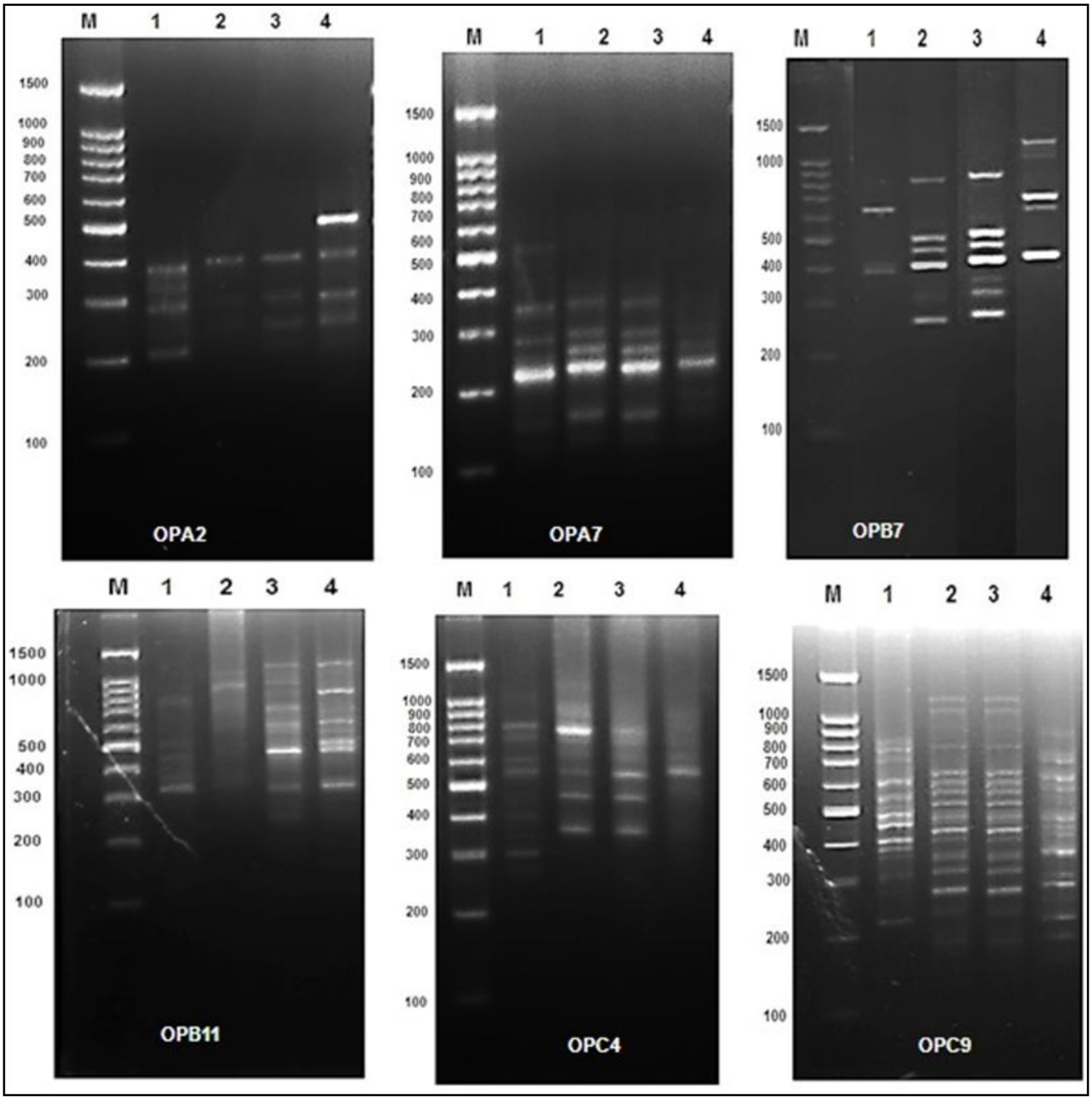

Plate 1: RAPD-PCR product profiles of walnut and almond genotypes.
M: DNA marker. 1: walnut. 2: sweet almond (scrub). 3: sweet almond (hard). 4: bitter almond.

Table 3: Bands characteristics produced by molecular markers (RAPD& ISSR) in walnut and almond genotypes.

Marker	Primer name	Total bands	Monomorphic bands	Polymorphic Bands	%Polymorphism
RAPD	OP-A2	6	3	3	50%
	OP-A07	8	4	4	50%
	OP- B7	11	1	10	90.91%
	OP-B11	9	4	5	55.56%
	OP-C04	11	4	7	63.64%
	OP-C9	18	11	7	38.89%
ISSR	14A	10	2	8	80%
	49A	6	2	4	66.67%
	HB-9	11	4	7	63.64%
	HB-10	16	2	14	87.5%
	HB-12	13	3	10	76.92%
	HB-15	12	4	8	66.67%

for OPC9 primer produced two unique bands. One band characteristic for almond genotype at 850bp, while other unique band specific for bitter almond genotype at 400 bp. Primer OPA2 gave one unique band for bitter almond at 500bp, while OPA7 produce one band characteristic for walnut genotype as unique band at 500 bp and primer OPB11 generated one unique band for walnut genotype at 400bp as illustrated in Plate 1.Higest percentage of polymorphism produced by RAPD primers was generated by primer OPC9 as 38.89%, while the lowest percentage of polymorphism produced by OPB7 as 90.91% as shown in table 2.

Table 4: Similarity coefficient between walnut and almond genotypes using RAPD marker.

Genotypes	1	2	3	4
1	1			
2	0.083	1		
3	0.071	0.832	1	
4	0.351	0.066	0.12	1

Table 5: Similarity coefficient between walnut and almond genotypes using ISSR marker.

Genotypes	1	2	3	4
1	1			
2	-0.077	1		
3	-0.156	0.864	1	
4	-0.317	0.339	0.329	1

Table 6: Similarity coefficient between walnut and almond genotypes using RAPD and ISSR markers.

Genotypes	1	2	3	4
1	1			
2	-0.003	1		
3	-0.052	0.85	1	
4	-0.011	0.219	0.238	1

1: walnut. 2: sweet almond (scrub). 3: sweet almond (hard). 4: bitter almond.

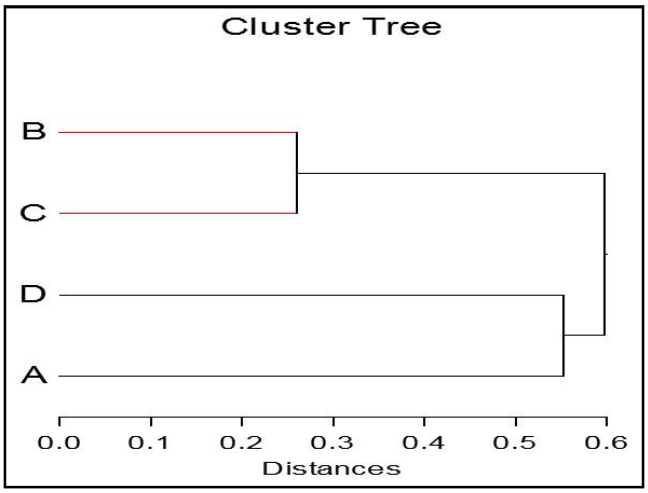

Fig. 1: Cluster analysis showing the relationships between walnut and almond genotypes by Distance metric is Euclidean distance Average linkage method using RAPD marker. A: walnut. B: sweet almond (scrub). C: sweet almond (hard). D: bitter almond.

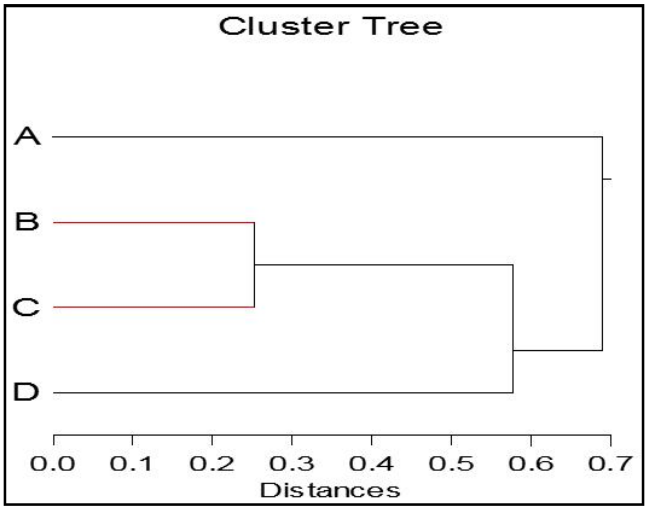

Fig. 3: Cluster analysis showing the relationships between walnut and almond genotypes by Distance metric is Euclidean distance Average linkage method using RAPD & ISSR markers. A: walnut. B: sweet almond (scrub). C: sweet almond (hard). D: bitter almond.

For ISSR analysis

Six ISSR primers were used to study genetic diversity among almond and walnut genotypes produced 68 total bands with 17 total bands and 51 polymorphic bands with molecular size varied from 100 to 1300 bp as appeared in table 3. ISSR-PCR product profiles gel showed in Plate 2. HB10 primer showed highest number of total bands as 16 bands with two monomorphic bands and 14 polymorphic bands. Primer 49A generated lowest number of total bands as six bands with two monomorphic bands and four polymorphic bands. Primer HB10 produced highest percentage of polymorphism among ISSR primers

as 87.5%, where HB9 primer generated lowest percentage of polymorphism as 63.64% as recorded in table 2. Primer HB10 generated highest number of unique bands as six bands, four unique bands for bitter almond at size bands 150, 190, 250 and 350 bp and two unique bands characteristic for walnut genotype at bands with molecular size of 1600 and 1700 bp.

Primer HB9 generated three unique bands specific for walnut genotypes at molecular size of 400, 1200 and 1300 bp. Primers HB12 and HB15 gave two unique bands for each primer characteristic for walnut genotype at molecular size of 800 & 1000 bp for HB12 primer and molecular size of 800 & 900 bp for Hb15 primer. Primer 14 A produced one unique band for walnut genotype at 800 bp and one unique band for sweet almond (hard almond) at 100 bp. Primer 49 A produced one unique band characteristic for bitter almond at 1100 bp and one unique band for walnut genotype at 700 bp as illustrated in gel profile in Plate 2. Comparative percentage of polymorphism among RAPD and ISSR molecular marker

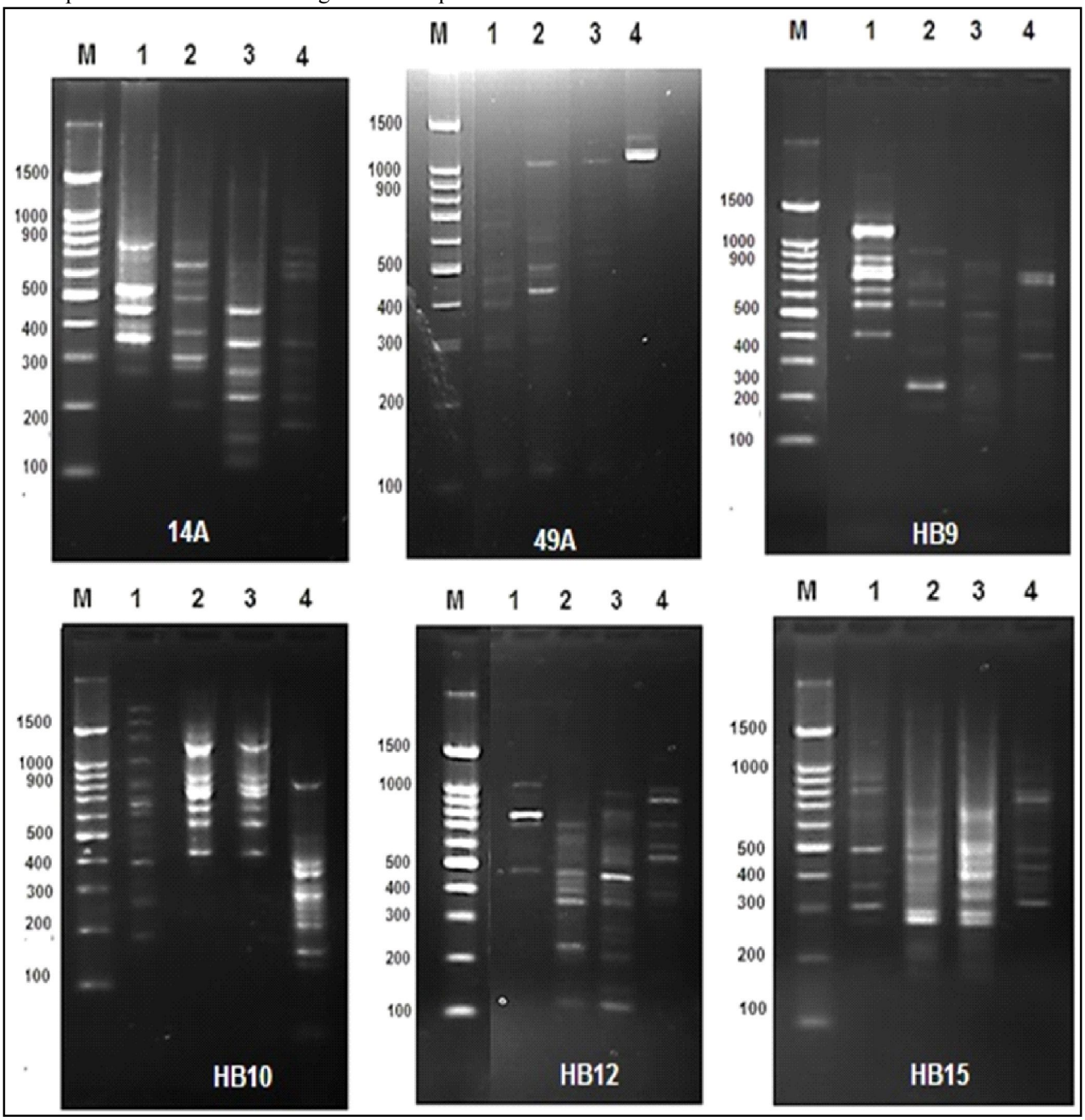

Plate 2: ISSR-PCR product profiles of walnut and almond genotypes.
M: DNA marker. 1: walnut. 2: sweet almond (scrub). 3: sweet almond (hard). 4: bitter almond.

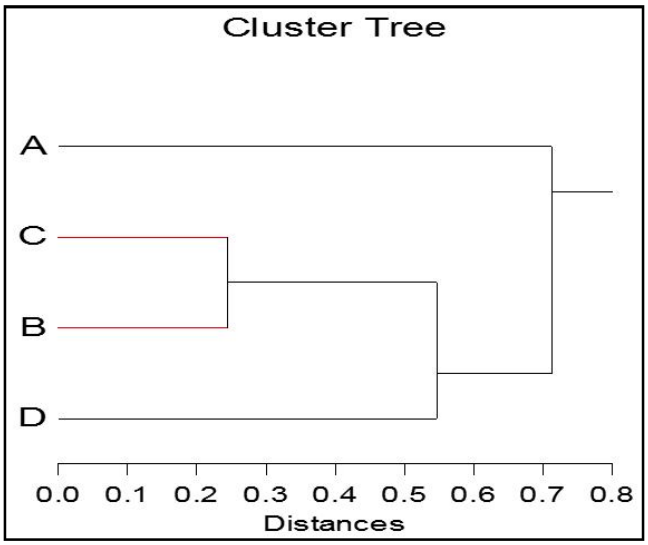

Fig. 2: Cluster analysis showing the relationships between walnut and almond genotypes by Distance metric is Euclidean distance Average linkage method using ISSR marker. A: walnut. B: sweet almond (scrub). C: sweet almond (hard). D: bitter almond.

was recorded in table 3, showed highest polymorphism for ISSR primers was 75%.

Data analysis

Cluster analysis was conducted based on RAPD analysis, generated dendrogram divided into two groups first group includes walnut genotype and bitter almond genotype, where the second group includes sweet almond (hard and scrub almond) as presented in Fig. 1.

On the other hand similarity distance showed highest similarity was 0.832 between sweet almond (hard) and sweet almond (scrub), while the lowest similarity was 0.066 among sweet almond (scrub) and bitter almond as represented in table 4.

Cluster analysis was conducted to show genetic diversity among studied genotypes based on ISSR primers produced dendrogram divided into two groups, first group include walnut genotype only, second group includes almond genotypes (sweet and bitter) in Fig. 2.

Similarity genetic distance based on ISSR primers was highest 0.864 between sweet almond (hard) and sweet almond (scrub), while the lowest was -0.077 between walnut genotype and sweet almond (scrub) as illustrated in table 5.

Cluster analysis generated dendrogram based on RAPD and ISSR molecular markers was illustrated in Fig. 3 separated unto two groups, one group contains walnut genotype only, second group contains sweet almond and bitter almond, where the second group also divided into two subgroups, one subgroup includes two genotypes of sweet almond (scrub & hard) and other subgroup includes only bitter almond.

Genetic distance similarity based on RAPD and ISSR primers was presented in table 6. Highest similarity was 0.85 between sweet almond (hard) genotype and sweet almond (scrub) genotype. Lowest similarity was -0.003 between walnut genotype and sweet almond genotype (scrub).

Discussion

Improving the species and production of new lines and cultivars are in relation with studying genetic diversity and species distribution that produce valuable information to breeding programs and conservation of genetic resources which associated with food production in agriculture (Tabasi *et al*., 2020). Many farmers cultivated different cultivars and lines from walnut and almond in temperate regions due to their nutritive importance and economical value, so these types of fruits considered as important fruit trees. Estimation of genetic diversity for both wild and cultivated plants depends on molecular markers with different ploidy levels (Aliyev *et al*., 2007). Evaluating phylogenetic relationships among studied species and genetic fingerprinting by using molecular markers as RAPD and ISSR are efficient tools in conservation and breeding programs (Mahmoodi *et al.,* 2012). Six RAPD primers analysis produced 63 total amplified bands and percentage of polymorphism was 57.14% was higher than percentage of polymorphism produced by RAPD marker studied by Fakhraddin *et al.,* (2013) was observed in walnut using RAPD marker. The 63 total bands with 27 common bands and 36 polymorphic bands were higher than the result of Nicese *et al.,* (1998) and Potter *et al.,* (2002).

ISSR molecular marker are helpful in fields of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of plant species (Jabbarzadeh *et al*., 2010). They have been effectively used in many tree species, including walnut (*J. regia* L.*)* (Pollegioni *et al*., 2003), olive (*Olea europaea* L.*)* (Terzopoulos *et al.,* 2005), mulberry (*Morus* L.) (Vijayan *et al.,* 2006) and plum (*Prunus* L. spp.) (Liu *et al.,* 2007). For ISSR marker, six primers produced 68 total bands with 17 monomorphic bands and 51 polymorphic bands illustrated genetic diversity among walnut and almond genotypes. This result in agreement with Chatti *et al*., (2010) who studied genetic variability among Tunisian *Ficus* tree cultivars using 48 ISSR markers and detect polymorphism among them. ISSR marker generated 17 unique bands among almond and walnut genotypes was lower than unique bands (19) generated from nine ISSR were used to study genetic

diversity among 18 almond genotypes (Abodoma *et al*., 2017). Cluster dendrogram in addition to similarity correlation among walnut and almond genotypes separated studied genotypes into two groups one group and has highest similarity among two sweet almond genotypes (hard & scrub) and other group with lowest similarity between walnut and sweet almond genotypes in agreement with the result of Shah *et al*., (2019). ISSR marker produces higher similarity 75% than RAPD marker 57.14%, so ISSR molecular markers gave efficient result in diversity among species than RAPD markers with regards to polymorphism detection using in genetic diversity between walnut and different almond genotypes. This validates that ISSR markers are useful markers in genetic divergence studies allowing an unequivocal identification for genotypes. The high level of polymorphism possibly reflects the outcrossing character of walnut because almost similar results have been obtained in fruit and nut tree species like pistachio (Ji *et al*., 2014).

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